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2. Patent application number *(The Patent Office will fill in this part)*	**9711964.8** -9 JUN 1997

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Medical Research Council

20 Park Crescent
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United Kingdom

Patents ADP number *(if you know it)*

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SQ6007001

4. Title of the invention

LIVE ATTENUATED VACCINES

5. Name of your agent *(if you have one)*

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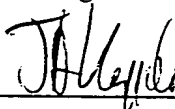   to grant of a patent *(Patents Form 7/77)* - -

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LIVE ATTENUATED VACCINES

The present invention relates to attenuated strains of pathogenic bacteria, particularly of *Neisseria meningitidis*. These strains are particularly useful as live attenuated vaccines.

5

Bacterial infections are a significant cause of disease throughout the world. Many bacterial infections can prove fatal, particularly in vulnerable individuals such as infants, children, the elderly or those who are immunocompromised by other diseases or through injury.

10 Bacterial diseases which are still prevalent include meningococcal meningitis, caused by *Neisseria meningitidis* and gonorrhoea, caused by the related organism *Neisseria gonorrhoeae*. Both these organisms are gram-negative bacteria. Other gram-negative bacteria include *Helicobacter pylori* (associated with stomach ulcers), *Salmonella spp.*, e.g. *Salmonella typhi* or *Salmonella typhimurium*, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), 15 enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), verotoxigenic *E. coli* (VTEC), *Vibrio cholerae*, *Shigella spp.*, (all of which cause gastric infections), *Haemophilus influenzae* and *Bordetella pertussis*, which may cause meningitis and whooping cough respectively, particularly in children, and *Pseudomonas aeruginosa*, which can cause infection in infants, children and adults and is particularly a problem in individuals who have cystic 20 fibrosis.

The goal of any vaccine development programme is to provide a vaccine which is safe, capable of providing cross-group protection and capable of providing long-term protection.

25 Traditionally there are three main groups of vaccine types. (1) Killed vaccines, where the killed pathogen is used directly, (2) Defined vaccines where purified components of the pathogen are used and (3) Live attenuated vaccines, where the live pathogen is used which has been rendered unable to grow for extended periods in the host. Each of these vaccine types presents different advantages and disadvantages.

30

Killed vaccines are superficially attractive, particularly from the safety point of view. However such vaccines can not proliferate or colonise, nor is there any dynamic gene expression, which

might result in the production of useful antigens which elicit an immunoprotective response. Indeed the production of the killed vaccine usually results in the exposure of internal antigens such as lipid A which may compromise its use due to induction of adverse reactions in the host. In most cases the cause(s) of these deleterious effects are unknown.

5

In view of such problems, many researchers have endorsed a defined or subunit vaccine approach where those antigens which appear to elicit the "best immune response" in the host are purified and used directly as a vaccine. This approach again avoids the use of live bacteria which is also attractive from the safety and regulatory point of view. The chosen components

10 may be subunits of actual virulence proteins or somatic proteins. There are obvious advantages to this approach:- (1) The vaccine is totally defined and the immunogenicity of each purified component protein is understood. (2) Any toxic component which may elicit adverse effects can be eliminated from the vaccine. (3) If the same strain and procedures are used there is the potential for vaccines made in different locations being consistent. (4) Since the vaccine is

15 composed of purified cell components, it is likely to be relatively stable under normal conditions of storage.

There are also major disadvantages to this approach. (1) Only selected cell components are used and these may not on their own be sufficient to elicit good protective long term immunity.

20 (2) The most important antigens for full protection may be unidentified and/or only be expressed under *in vivo* conditions and therefore will be absent from the vaccine. (3) Vaccine production entails expensive protein and/or cell component purifications reliant on advanced technology. (4) Protein antigens most important for the host immune response will most probably be secreted toxins or surface exposed outer membrane proteins which may undergo

25 some degree of denaturation during purification. Denatured proteins will lose conformational epitopes that may be important and if used in vaccines may elicit immune responses to epitopes which are not present in the native, membrane associated protein. (5) Purified outer membrane proteins may require an additional, expensive renaturation step, and possibly re-insertion into a membrane vesicle carrier to make them useful as a vaccine. (6) Renaturation of proteins *in vitro*

30 is difficult and never completely successful. Aberrant epitopes may remain and the important epitopes of the protein not be reformed even when inserted into membrane vesicles. Therefore it is probably naive to expect that a few proteins purified apart from their normal membrane

environment would elicit a good cell mediated response in the host. (7) Association of individual outer membrane proteins in the membrane surface of the intact organism appears important for the induction of an immune response to the individual proteins. Proteins removed from such associations may not elicit protective/bactericidal responses even though they may do

5 so in the intact organism. This may be due to conformational epitopes being formed between two or more associated proteins. (8) The normal association of proteins within the membrane may generate important antigenic epitopes which could be lost when individual components are purified. Thus in many respects, defined/subunit vaccines are far from ideal.

10 Live attenuated vaccines offer the best prospect for a vaccine which will give similar protection to that seen with the natural disease. The advantages of a live attenuated vaccine are numerous. (1) The whole live organism is used, rather than dead cells or selected cell components which may exhibit modified or denatured antigens. Therefore the potential to elicit a good protective long term immunity should be higher. (2) The most important antigens for full protection need

15 not be identified, since the vaccine will only have specific modifications for attenuation it will still be able to express these important antigens under *in vivo* conditions. (3) Production costs should be a fraction of more intensive and expensive vaccine production procedures, since there is no protein/subunit purification, protein refolding, or down stream processing required except the minimum essential for the vaccine application. (4) Protein antigens in the outer membrane

20 will maintain their tertiary and quaternary structures. (5) The associations between individual proteins in the membrane surface of the vaccine strain which may be important for the generation of conformational epitopes between two or more associated proteins will be maintained. (6) The fluid structures of these proteins will also be maintained in the attenuated vaccine strain. (7) Since the vaccine is live it will display dynamic gene expression in response

25 to environmental changes and express those proteins which are only present or enhanced *in vivo* in the wild type pathogen. (8) The host responses to the attenuated strain would mimic that expected for the pathogen since the attenuation process would not affect the expression of the important immunogenic proteins. (9) Production of live attenuated vaccines is much simpler. As long as recommended growth conditions are adhered to and a standard stock of the vaccine

30 strain is maintained and used as the initial inoculum vaccines should be uniform in composition and efficacy. (10) With improved methods of freeze drying and storage the vaccine should be stable and viable at normal ambient temperatures. (11) Live attenuated vaccines can in

addition, be important vehicles for the delivery of other antigens/immunogens for example toxins. This would be particularly important where the immunogen which confers complete protection is a secreted toxin, e.g. diphtheria toxin, pertussis toxins or cholera toxin B subunit.

5 However, one difficulty with the production of live attenuated vaccines is that during bulk culture the organism does not always express some proteins which are important for host protection. In addition, once such vaccines have been administered to the host, the vaccine strain does not have the optimum time or metabolic resources during its limited colonisation to express these protein *in vivo* to a sufficient level to confer protection.

10

Summary of the Invention.

The present invention is based on the finding that altering the regulation of the ferric uptake regulation (*fur*) gene in *N. meningitidis*, such that its expression is independent of the iron 15 concentration in the environment of the bacterium, enhances the expression of important protective antigens when the bacterium is grown in culture. This has important implications for the manufacture of live vaccines since the increased expression of these protective antigens during the manufacture process will increase the efficacy of the live vaccine when administered to an animal or human subject.

20

Thus in a first aspect the invention provides an attenuated bacterium in which the native *fur* gene, or homologue thereof, is modified such that the expression of the *fur* gene product, or homologue thereof, is regulated independently of the iron concentration in the environment of the bacterium. The attenuated bacterium is preferably a gram-negative bacterium, and more 25 preferably selected from the group consisting of *N. meningitidis*, *N. gonorrhoeae*, *Helicobacter pylori*, *Salmonella spp.* (including *S. typhi* and *S. typhimurium*), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) enterohaemorrhagic *E. coli* (EHEC), verotoxigenic *E. coli* (VTEC), *Vibrio cholerae*, *Shigella spp.*, *Haemophilus influenzae*, *Bordetella pertussis* and *Pseudomonas aeruginosa*. Especially preferred are 30 *N. meningitidis* and *N. gonorrhoeae*, most preferably *N. meningitidis*.

Preferably, the bacterium has been attenuated by mutation of a gene essential for the production

of a metabolite or catabolite not produced by a human or animal. The mutated gene is most desirably an *asd* gene or a gene of the *aro, pur,* or *pyr* pathways such as *aro*A. When the bacterium is *N. meningitidis* or *N. gonorrhoeae,* it may also carry a *gal*E mutation. Optionally the *gal*E mutation is accompanied by modification or elimination of expression from the *opc*

5 gene.

In yet another preferred aspect, the bacterium further comprises a *rec*A mutation. Optionally, in addition to or instead of the *rec*A mutation, the bacterium also comprises a mutation in a gene responsible for the uptake of DNA. These gene mutations will provide attenuated strains in

10 which the possibility of homologous recombination with DNA from wild-type strains has been minimised. Such a gene may include the *com*A pilin gene of *N. gonorrhoeae* (Molecular Microbiology (1993) **10**: 699-712) or its homologues in other species - particularly *N. meningitidis* - which may be obtained by methods analogous to those described herein for other genes.

15

Further modifications may be made for additional safety. For example the bacterium may additionally comprise a mutation in which expression of toxin genes has been modified or eliminated. In particular, for *Neisseria* species, the RTX (repeats-in-toxin) toxin gene may be modified or eliminated. This is accomplished by deleting the *frp*A gene in the *frp* polycistronic

20 locus, while maintaining the correct reading frame for the polycistron.

Certain bacteria such as *N. meningitidis* and others mentioned above are known to produce membrane vesicles. This is caused by aberrant cell division resulting in DNA-less cells (or mini-cells) which bud from the dividing bacteria. Such membrane vesicles, particularly of

25 *N. meningitidis,* form a further aspect of the invention. Production of such vesicles is enhanced by mutation in the locus homologous to the *E. coli minA/min*B locus and thus in a further aspect of the invention mutations in these genes may be made.

Particularly preferred strains of *N. meningitidis* have the genotypes: Δ*asd,* Δ*gal*E, *lac:fur* fusion,

30 Δ*rec*A; or Δ*aro*A, Δ*gal*E, *lac:fur* fusion, Δ*rec*A. In either case the strains may further comprise at least one of a *minA/*B mutation, an RTX negative phenotype, and modified expression of the *opc* gene.

The invention further provides a vaccine preparation which comprises a bacterium or a vesicle preparation of the invention together with a pharmaceutically acceptable diluent or carrier. Such vaccines may be used in a method of treatment of the human or animal body. Such a method may comprise protecting an individual against a bacterial infection by administering to an individual an effective amount of a vaccine composition comprising an attenuated bacterium, according to the present invention, normally associated with said infection.

The invention further provides a process for preparing a vaccine composition comprising an attenuated bacterium according to the invention which process comprises (a) inoculating a culture vessel containing a nutrient medium suitable for growth of said bacterium; (b) culturing said bacterium; (c) recovering bacteria from the culture and (d) mixing said bacteria with a pharmaceutically acceptable diluent or carrier.

The present invention also provides a method for producing a bacterium according to the invention which method comprises modifying the native *fur* gene, or homologue thereof, of an attenuated bacterium such that expression of said *fur* gene or homologue is regulated independently of the iron concentration in the environment of the bacterium. Preferably, said bacterium has been attenuated by mutation of at least one gene essential for the production of a metabolite or catabolite not produced by a human or animal, more preferably by a mutation of an *asd* gene and/or a gene of the *aro*, *pur*, or *pyr* pathways.

Brief Description of the Drawings.

Figure 1 illustrates the production of an *asd* mutant according to the present invention.

5   **Figure 2** illustrates the production of a *fur/lac* fusion strain according to the present invention.

Detailed Description of the Invention.

The term "vaccine" as used herein means an agent used to stimulate the immune system of a
10 vertebrate, particularly a warm-blooded vertebrate including humans, so as to provide protection
against future harm by an organism against which the vaccine is directed. The immune system
can provide two types of responses to foreign organisms - cell-mediated (T cell) and humoral
(B cell/antibody) responses. Both types of responses can be stimulated by a suitable vaccine to
produce a primary immune response which leads to the development of immunological
15 memory, providing protection against a subsequent infection by a pathogen. Stimulation of the
cell-mediated immune system results in the development of naive T cells into effector T cells
which are able to react much more quickly to future challenges. Stimulation of B cells of the
humoral immune system leads to the production of antibodies, desirably neutralising antibodies/
bacteriocidal antibodies, directed to epitopes found on or in the attenuated bacterial strain. The
20 antibody so produced may be any of the immunological classes, such as the immunoglobulins
A, D, E, G or M. Vaccines which stimulate the production of IgA are of particular interest since
this is the principle immunoglobulin produced by the secretory system of warm-blooded
animals, and the production of mucosal antibodies will help prevent infection or colonisation of
the nasopharynx, which is one natural route of infection of bacteria including *N. meningitidis*.
25 However, this and other organisms may also be infectious via the bloodstream and thus an IgM
and IgG response will also be desirable.

The bacterium of the invention may be in isolated form. This is usually desirable when the
bacterium is to be used for the purposes of vaccination. The term "isolated" means that the
30 bacterium is in a form in which it can be cultured, processed or otherwise used in a form in
which it can be readily identified and in which it is substantially uncontaminated by other
bacterial strains, for example non-attenuated parent strains or unrelated bacterial strains. The

term "isolated bacterium" thus encompasses cultures of a bacterial mutant of the invention, for example in the form of colonies on a solid medium or in the form of a liquid culture, as well as frozen or dried preparations of the strains.

5 The term "mutation" and the like refers to a genetic lesion in a gene which alters the levels of expression of the gene or the activity of the gene product. Such a mutation may exert its effect on the levels of expression of a gene product at any stage from the level of transcription to the level of translation and protein stability. Alternatively, a mutation may cause in a change in the coding sequence such that the resulting gene product has altered activity, generally reduced 10 activity. For example, an "inactivating mutation" would render the gene non-functional. The term "inactivating mutation" thus envisages deletion of the entire gene or substantial portions thereof, and also point mutations in the coding sequence which result in truncated gene products unable to carry out the normal function of the gene. Where deletion of a portion of a gene is carried out, it may be desirable to introduce frame shift mutations downstream of the deleted 15 region to remove the possibility of small sections of the gene being expressed.

A mutation introduced into a bacterium of the invention will generally be a non-reverting attenuating mutation. Non-reverting means that for practical purposes the probability of the mutated gene being restored to its normal function is small, for example less than 1 in 10^6 such 20 as less than 1 in 10^9 or even less than 1 in 10^{12}. However despite this, it may be considered desirable that a bacterium for use as a live attenuated vaccine should carry at least two attenuating mutations. Usually these mutations will be in separate genes.

An "attenuating mutation" is a mutation in a gene which is required for the growth of a 25 bacterium. Such a gene is thus usually a metabolic, catabolic or biosynthetic gene. Preferred attenuating mutations are to genes which are required for the production of an essential nutrient or intermediate which is not available in the tissues of the human or animal host in which the bacterium is normally pathogenic. Thus the attenuated bacterium will not be able to simply obtain the essential nutrient or intermediate from the infected host.

30

A bacterium of the invention will be an attenuated form of a pathogenic bacterium. The term "pathogenic" means that the bacterium is capable of causing disease in a human or animal

(especially avian or mammalian, including human) host, although in some cases the bacterium may be carried asymptomatically by healthy individuals. Examples of the latter type include *N. meningitidis* and *S. aureus*.

5 Production of Bacteria.

The survival of any bacterium in a given environment depends on its ability to obtain all its growth requirements in competition with other resident organisms. It must express those proteins which are essential for growth and replication if it is to be competitive and survive.
10 Strains which carry a particular mutation or combination of mutations may be made from a species of bacteria in accordance with the present invention. These strains will carry particular genetic lesions or mutations which render the organism auxotrophic for an essential nutritional requirement. If the organism is unable to acquire this nutrient from its environment it will not proliferate and will die once endogenous levels are exhausted. If such mutations are
15 incorporated into normally pathogenic bacteria and the essential nutrient or its intermediates are absent from the tissues of the host then that mutant organism will be attenuated and unable to establish a normal infection, if colonisation is a component of the infection process.

Bacterial strains according to the invention may be constructed using recombinant DNA
20 methodology which is known *per se*. In general, bacterial genes may be mutated by a process of targeted homologous recombination in which a DNA construct containing a mutated form of the gene is introduced into a host bacterium which it is desired to attenuate. The construct will recombine with the wild-type gene carried by the host and thus the mutated gene may be incorporated into the host genome to provide a bacterium of the present invention which may
25 then be isolated.

Various assays are available to detect successful recombination. In the case of attenuations which mutate a target gene necessary for the production of an essential metabolite or catabolite compound, selection may be carried out by screening for bacteria unable to grow in the absence
30 of such a compound.

The mutated gene may be obtained by cloning the wild-type gene from the bacteria which is to

be attenuated, or from a closely related species, and manipulating it by standard recombinant DNA techniques. These include introducing deletions into the gene, e.g. by digesting with a restriction enzyme which cuts the coding sequence twice to excise a portion of the gene and then religating under conditions in which the excised portion is not reintroduced into the cut

5   gene. Alternatively frame shift mutations, which alter the reading frame of the DNA sequence, may be introduced by cutting with a restriction enzyme which leaves overhanging 5' and 3' termini, filling in and/or trimming back the overhangs, and religating. Similar mutations may be made by site directed mutagenesis. These are only examples of the types of techniques which will readily be at the disposal of those of skill in the art.

10

The cloning of the wild-type gene may be undertaken using PCR cloning methods which are also known *per se* in the art. Such methods require knowledge of at least part of the sequence of the gene which is to be mutated. The sequence may be obtained by reference to databases such as Genbank. Where a gene sequence is not known for a particular organism, consensus

15   sequences may be determined by aligning sequences of corresponding genes of other, related, organisms and determining regions which are most conserved. In general, many genes, especially those of metabolic and catabolic pathways are well conserved between gram-negative bacteria and appropriate PCR primers can be determined without difficulty.

20   The above techniques are exemplified further in the accompanying examples and can be readily adapted to provide other embodiments of the invention. Reference may also be made to, for example, Sambrook *et al.*, "Molecular Cloning - A Laboratory Manual" (Cold Spring Harbor, 1989) or "Short Protocols in Molecular Biology - A Compendium of Methods from Current Protocols in Molecular Biology" eds. Ausubel *et al.* (John Wiley & Sons, 1992).

25

Attenuating Mutations.

The invention provides, in a particular preferred embodiment, a live attenuated vaccine which is effectively attenuated by construction of specific deletion and frame-shift mutations initially in

30   the gene for aspartate semi-aldehyde dehydrogenase (*asd*). The *asd* gene is described further in Gene (1993) **129**: 123-128.

A lesion in the *asd* gene, encoding the enzyme aspartate β-semialdehyde dehydrogenase would render the organism auxotrophic for the essential nutrient diaminopimelic acid (DAP). Since this compound is an essential constituent of the cell wall for gram-negative and some gram-positive organisms and is absent from mammalian or other vertebrate tissues, mutants would

5 undergo lysis after about three rounds of division in such tissues. Such a mutation will limit the proliferation of the bacteria in host tissues but will not affect their growth during vaccine production since the essential nutrient DAP can be provided exogenously during bulk culture of the vaccine strain.

10 Other genes essential for the production of a metabolite or catabolite not produced by the human or animal subject include:

*aro*A. This gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which produces p-amino-benzoic acid (pAB). Mutations in this gene render the mutants deficient in

15 aromatic amino acid biosynthesis. After 3-4 rounds of division in a host (where the intermediate pAB is absent) protein synthesis will cease and the organism will eventually die and lyse. This *E. coli* gene is disclosed in Nature **291**: 238-239.

Mutation of other genes in the *aro* synthetic pathway is also possible. These may be made

20 instead of or in conjunction with the *aro*A mutant. These include the *aro*C and *aro*E genes. Such mutations are disclosed in EP-A-322 237.

pur - Mutations in any of the genes in the *pur* pathway (e.g. *pur*A or *pur*E) will render the organism auxotrophic for purines, essential constituents of nucleic acids. Many of the

25 intermediates of the pathway are unavailable in mammalian or other vertebrate tissues.

pyr - Mutations in any of the genes in the *pyr* pathway will render the organism auxotrophic for pyrimidines, essential constituents of nucleic acids. Many of the intermediates of the pathway are unavailable in mammalian or other vertebrate tissues.

30

Vaccine strains using deletions in these genes may also be employed either as alternatives to the *asd* gene or in conjunction with *asd* mutations. A double or triple mutant may be useful from

the safety point of view since it will be more attenuated, but such a double mutant may also be less efficient as a vaccine. Strains with different levels of attenuation may be assessed during trials for their degree of efficacy and any side effects. Constructs and mutations of these genes may be accomplished in a similar way as that described for *asd*.

5

Mutation to maximise exposure of important epitopes.

The *galE* gene codes for UDP-galactose-4-epimerase. In *Neisseria* this enzyme does not appear to have a role as part of the galactose biosynthesis pathway, but it is involved in the

10 incorporation of galactose into the phase-variable oligosaccharide side chains of LPS/LOS. A mutation in the *galE* gene denudes and shortens the LPS of galactose residues. Thus, a *galE* deletion exposes the constant region of the LPS to the immune system, which leads to the generation of a cross-protective response. The reduction in the length of LPS may reduce the capsule on the surface of the organism. A further effect of a *galE* mutation and the resulting

15 short LPS is that outer membrane proteins exposed on the cell surface will become more accessible to the hosts immune system potentially leading to an improved immune response to the vaccine strain. Thus incorporation of a *galE* mutation, rendering the gene non-functional, into a *Neisseria* strain of the invention is preferred.

20 In addition, without the incorporation of galactose into the LPS the potential autoantibody-producing epitopes of LPS will not be incorporated. These epitopes are normally found on host epithelial cells and in many host secretions. Thus their removal from vaccine strains will greatly reduce the danger of immunopathological effects of the vaccine.

25 It has also been shown in bacteria, particularly *N. meningitidis*, that the *galE* mutation results in an increase in *opc*-mediated invasion. For a vaccine intended for nasal delivery, this may be advantageous in stimulating the initial host tissue response and subsequent mucosal immunity, while other mutations (such as in the *asd* gene) would limit the proliferation of invading bacteria to approximately three rounds of division, precluding the possibility of a systemic

30 infection. Also there is some evidence that *galE* mutants are serum sensitive and therefore would be rapidly killed if they entered the hosts circulatory system.

N. meningitidis contains two chromosomal *galE* gene sequences, *galE1* and *galE2* at separate loci. *galE1* is complete, expressing a functional gene product, while *galE2* is truncated, and lacks homology over the first third of the protein. The *galE* gene and its role in biosynthesis of LOS/LPS is described in Molecular Microbiology (1993) **10**: 361-369.

Invasion/adhesion.

A *galE* mutation will expose the Opc protein thus making the vaccine strain able to colonise and invade into the blood stream more efficiently. This would be important to get a good mucosal and serum immune response in the limited time that the strains survive in the human body. There are two possible modifications to *opc* that may be made in accordance with one embodiment of the invention.

If there is excessive adhesion or invasion with the vaccine strain, then *opc* expression may be modified or even eliminated entirely. Where *opc* expression is found to be important for vaccine efficacy but is variable in expression, then *opc* may be placed under the control of another promoter e.g. *cat*, which can be more readily controlled. The *opc* opacity protein may be found by reference to the EMBL/GenBank/DDBJ databases. 1993. Accession number Z14063 (Microb. Pathog (1991) 11:249-257).

Mimicking *in vivo* expression of iron regulated proteins.

One failing of live attenuated vaccines in the past has been that during bulk culture the organism has not expressed some proteins which are important for host protection. Such proteins include iron regulated proteins, the production of which is regulated by the *fur* (ferric uptake regulation) gene. In addition, once such vaccines have been administered to the host, the vaccine strain has not had time or metabolic resources during its limited colonisation to express these proteins *in vivo*.

To overcome these problems, the present invention provides vaccine strains which have been modified such that many of the proteins which are likely to be important immunogens, e.g. the iron regulated proteins, are already expressed on the cell surface during bulk culture. This is

achieved by altering the regulation of the ferric uptake regulation (*fur*) gene (EMBL/GenBank/DDBJ databases. 1993. Accession number L19777, Mol. Microbiol (1994) 11(4): 725-737 (*Neisseria*); Mol. Gen. Genetics (1985) 200: 110-113 (*E. coli*)).

5   The *fur* gene may be modified to produce its gene product independently of the iron concentration in the environment of the bacteria by any suitable means. In this context, the term "gene" is intended to encompass both the coding sequence of the fur protein together with any transcriptional/translation control sequences present, including promoters, transcriptional stop sequences, ribosome binding regions and other translation regulation elements. Thus,

10   modifications may be made to the coding sequence and/or the transcriptional/translation control sequences. For example, the promoter sequences of the gene may be determined (e.g. by reference to the database sequence and/or sequencing this region using PCR primers directed upstream from the coding region) and the promoter regions responsible for iron concentration-dependent regulation may be modified by mutation, including deletion, insertion and/or

15   substitution. One such identified promoter region is the fur box. Since fur expression may be required for positive regulation of some essential genes in *Neisseriacae* and complete inactivation of the *fur* gene is often lethal to the bacterium, the minimum levels of expression from the modified *fur* gene will preferably be sufficient to allow viable growth of the bacterium. In particular, levels of expression from the modified fur gene are preferably at least equal to

20   those obtained using the native *fur* gene in the presence of low levels of ferric ions, i.e. the maximal repressed state.

Alternatively the endogenous *fur* gene may be placed under the control of another promoter entirely. Such a heterologous promoter may be any promoter which is regulated independently

25   of iron concentration and which functions in the host bacterium. Preferably said promoter comprises a bacterial promoter such as a promoter derived from that bacterium or a member of the same family, or from a bacteriophage. Suitable promoters include both constitutive promoters such as that of the chloramphenicol resistance gene and regulatable promoters such as the promoters of catabolic, metabolic and biosynthetic genes, including those involved in the

30   regulation of amino acid biosynthesis (which are induced when a required amino acid is absent or at a low concentration) or those involved in the metabolism of sugars (which are induced by the presence of the appropriate sugar). The *trp* promoter and the *lacZ* promoter are particular

examples of the above, and the latter is especially preferred.

The promoter region of the endogenous fur gene is conveniently modified by homologous recombination with a suitable construct. The construct may comprise all or part of the *fur* gene
5   together with the desired replacement promoter sequences provided that recombination results in modification of the endogenous *fur* gene. Alternatively, the endogenous *fur* gene may be inactivated, and a complete *fur* gene together with the desired replacement promoter sequences introduced into the bacterial genome or maintained as an extrachromosomal plasmid. This will ensure that even in the presence of iron little *fur* gene expression occurs, although the
10 heterologous promoter will allow sufficient expression of any essential genes positively regulated by fur.

In a particularly preferred embodiment of the invention, to overcome the problems of regulating *fur* and also to mimic *in vivo* levels of expression of iron regulated genes, *fur* gene expression is
15 placed under the control of the *lacZ* promoter/*lacI* repressor which confers positive regulation of

β-galactosidase in the presence of lactose. Preferably, the I^q mutation in *lacI* is included which increases the basal level of expression ten-fold. This provides for sufficient production of fur to allow expression of essential genes and also allows the control of fur expression to be regulated in response to intracellular lactose concentrations.
20

Mutations may also be made in the coding sequence of the *fur* gene, which encodes the fur protein. By way of example, a mutation can be made which modulates the affinity of the fur protein for ferric ions. Preferably, such a mutation would abolish or substantially reduce the affinity of the fur protein for ferric ions. In this way, the fur proteins transcriptional regulatory
25 functions would be independent of the iron concentration in the environment of the bacterium.

Preferably, an attenuated *asd* mutant is used and its *fur* gene replaced with a *fur* gene under the control of the *lacZ* promoter/*lacI* repressor. The resulting low levels of the fur protein would enable the negatively regulated fur-controlled genes to be switched on, mimicking iron
30 restricted conditions. The strain would therefore be fully attenuated but would fully express all iron-regulated proteins before being administered to the recipient without the need for iron-restricted growth conditions. The host would then be able to mount an immune response that

would mimic that in response to the wild-type pathogen.

Thus placing *fur* under the regulation of the *lacZ* promoter is preferred. This allows its expression only in the presence of lactose or one of the non-metabolisable lactose analogues

5 such as IPTG. Since the *lac* inducer dissociates from the *lac* operator in the presence of lactose, fur expression can be reduced down to basal levels by growing the strain in the absence of lactose. This effectively up-regulates all fur regulated genes mimicking growth in low iron, *in vivo* conditions. The result will be that in the absence of lactose, the bacteria will respond as if they are experiencing iron restricted conditions, producing the iron regulated proteins normally

10 expressed *in vivo*, without the use of large quantities of iron chelators. It is thus possible to control *fur* expression independent of the iron concentration in the medium. *Fur* expression will only be at "trickle through" basal levels in the absence of lactose. The maintenance of this basal level of expression is thought to be important for cell viability.

15 Therefore, in this embodiment of the invention, when the vaccine is administered to the host, many of the important proteins will already be present on the bacterial surface. This will relieve the metabolic burden that the bacterium will experience *in vivo*, freeing the resources of the bacterium to express other *in vivo* regulated proteins which are not iron regulated but are important for host protection.

20

The production of attenuated strains of bacteria in which the expression of *fur* has been modified as described above is a generally applicable technique. Thus any normally pathogenic bacterium which has a genome comprising a *fur* gene or homologue thereof, which bacteria is attenuated by deletion or modification of a gene essential for growth in a host in which the

25 bacteria is pathogenic, may be modified as described above so that it produces the *fur* gene product, or homologue thereof, independently of the iron concentration in the environment of the bacteria. *Fur* homologues can be identified by sequence comparison with other known *fur* gene sequences from, for example *E. coli* and *N. meningitidis*. Preferably, such homologues are substantially homologous to another known *fur* gene. In particular it is preferred that

30 homologues have sequence identity of at least 60% at the amino acid level, more preferably 70%, over a stretch of at least 100 amino acids. *Fur* homologues can also be identified on the basis of function. The product of the *fur* gene in *Neisseria* controls the expression of numerous

genes including those encoding toxins and iron uptake components, and is responsive to ferric ion concentrations. Thus genes identified in other bacteria which encode transcription factors which also have these ferric iron-responsive properties may be used.

5   **Mutations to achieve genetic isolation and stability.**

Another consideration of live attenuated vaccines which has to be addressed is the genetic stability of the strain. The strain must not be able to revert to a wild-type phenotype, nor must it lose the ability to express genes important for host protection. Genetic stability can be increased 10 by introducing a deletion in the recA gene. This gene enables the organism to repair its DNA by homologous recombination. The recA mutation knocks out homologous recombination - the process which is exploited for the construction of the mutations. Once the recA mutation has been incorporated the strain will be unable to repair the constructed deletion mutations. A mutation in recA will also make the strain sensitive to ultra-violet light and will be an important 15 containment factor.

In addition, it would prevent the strain from repairing the genetic lesions by homologous recombination using wild-type DNA found in the environment or the tissues of the host (commensal *Neisseria* for example). A recA mutation also increases the general stability of the 20 strain by reducing the loss of phenotypic characteristics by deletions and inversions which are mediated at least in part by such recombination pathways. *RecA* genes have been widely studied in the art and their sequences are available. Reference may be made to EMBL/-GenBank/DDBJ databases. 1992. ID code NM446REC (or Molecular Microbiology (1992) 6:2135-2146).

25

In many cloning procedures, selectable markers are used to select desired mutants. However, it may be preferred to remove subsequently the selectable marker. Since introducing a recA mutation will disable the recombination apparatus of the bacterium it will not be possible to use a subsequent recA-dependent recombination event to remove the selectable marker. Thus a 30 particularly preferred method for introducing a recA mutation which allows subsequent removal of a selectable marker is to flank the selectable marker sequences with sequences which are recognised by a recombinase/resolve-type enzyme. For example, the product of the *parA* gene

is a resolvase that specifically excises regions of chromosomal DNA flanked by 140 bp *res* sites (J. Bact. (1995) 177:52-58). Therefore the *recA* mutation cassette may comprise a selectable marker gene flanked by *res* sites. Once mutant bacteria with the desired *recA* mutation have been selected, introduction of a plasmid encoding the *parA* resolvase will result in excision of
5 the selectable marker gene. Plasmid RP4 (J. Bact. (1995) 177:52-58) is an example of such a plasmid. Expression of the resolvase protein even at low efficiency will be sufficient to result in the excision of the selectable marker gene flanked by the *res* sites. The plasmid bearing the parA gene lacks a Neisserial origin of replication and would be lost naturally from *Neisseria* sp.

10 **Additional genetic stability.**

Once the recA activity has been abolished, the bacterial strain may still have some residual ability to take up exogenous DNA. This may vary from strain to strain. If the frequency of such events is considered too high (e.g. by regulatory authorities) a further mutation may be
15 introduced to reduce DNA uptake. Such a mutation may be made in any gene involved in DNA uptake.

The *comA* gene of *N. gonorrhoeae* is involved in competence. A mutation in the *comA* locus would render this organism unable to take up exogenously added DNA, making the strain
20 genetically isolated, biosafe and more stable. Corresponding mutations at homologous loci may be made in *N. meningitidis* and other organisms. Those of skill in the art will appreciate that it will be necessary to recombine the *recA* and *comA* (or other gene involved in DNA uptake) constructs simultaneously, since both recombination and competence functions to introduce these lesions. Thus where a *recA* and/or *comA* lesion is required in a strain, this/these will be
25 introduced as the final genetic modification, following the other modifications described herein.

RTX toxin.

The *N. meningitidis* vaccine strains produced in accordance with the embodiments of the
30 invention described above will still express the genes for the RTX toxin and its secretion machinery (encoded by *frp* locus). The outer membrane proteins for toxin secretion may be important protective antigens. However, the toxin itself, which is iron regulated, may cause

some adverse reactions, for example in some subgroups of patients or with some attenuated strains. If the production of the RTX toxin causes problems during the limited colonisation of the vaccine strain, then the toxin gene will be inactivated by defined deletion. This will leave the genes for the outer membrane transport proteins intact, since they may be important elements

5   for the efficacy of the vaccine.

One option would be to inactivate the toxin structural gene (*frp*A) by the insertion of a *Sac*RB cartridge from *B. subtilis* . This would result in a sucrose sensitive phenotype which would provide a molecular tag, allowing genetic stability to be monitored during manufacture. The

10   RTX toxin gene may be found by reference to Mol. Micro (1993) 9:85-96.

Although an RTX mutation is envisaged primarily with reference to *N. meningitidis*, many other bacteria have a similar RTX toxin and corresponding mutations may be made in these. In addition, other bacteria produce other types of toxin and it may be necessary to introduce

15   mutations into the genes encoding these toxins, using standard techniques.

Membrane Vesicles.

In addition to the live attenuated vaccine, membrane vesicles derived from this *N. meningitidis*

20   or other vesicle-producing bacteria may be a better option for certain patients or patient groups. *N. meningitidis* and other bacteria naturally produce membrane vesicles which may be isolated. These may be used directly to induce a strong immune response.

Mutations in the *E. coli* *min*A/*min*B locus (see Eur. J. Biochem. (1981) 116:331-335) cause

25   aberrant cell division and the production of mini DNA-less cells. Mutations in this locus and homologous loci in other bacteria will greatly increase the production of membrane vesicles by these bacteria. Thus the present invention also provides a bacterium with additional mutations in loci homologous to the *E. coli* *min*A/*min*B locus.

30   **Strains of *N. meningitidis*.**

To provide attenuated strains of *N. meningitidis* any suitable starting strain may be used. In

general, clinical isolates are preferred since these represent strains which have been shown to cause disease in patients. Clinical isolates include KH 454 Serotype B15 P1.7 (J. Med. Microbiol. (1995) 42:353-361) and nm3474 Serotype B2B P1.10 (available from Nottingham Public Health Laboratory Service, Nottingham, UK). However these are only examples and

5 many clinical isolates are available in medical microbiology laboratories throughout the world.

Whatever starting strain is used, preferred genotypes of attenuated strains in accordance with the invention include the following:

10 1.   Δ*asd*, Δ*galE*, *lac:fur* fusion, Δ*recA*.

2.   Δ*aroA*, Δ*galE*, *lac:fur* fusion, Δ*recA*.

In either case one or more of the following characteristics are additionally preferred:

15 i)   a mutation in a homologue of *min*A/B

ii)   an RTX negative phenotype; and

iii)   an *opc* gene, the expression of which has been modified or eliminated.

N. meningitidis **as a carrier for other antigens.**

20

Attenuated strains of the invention will also be useful as a vehicle for the delivery of other antigens from other pathogens, including viral and bacterial pathogens. This will be useful where protection against such other pathogens can be obtained by stimulation of the immune system by such an antigen. Examples of this include the hepatitis B surface antigen, pertussis

25 toxin, *V. cholerae* or ETEC B subunit toxin or diphtheria toxin.

In these cases a gene encoding the antigen will be operably linked to a promoter capable of functioning in the bacterium of the invention to provide an expression cassette or vector and such a cassette or vector will be introduced into the bacterium. The cassette or vector may also

30 comprise genomic sequences from said bacterium to allow for homologous recombination of the cassette or vector into the genome of the cell. For example, the cassette or vector may be designed such that the antigen gene replaces one of the genes described above which is to be

attenuated.

Growth of strains and production of vaccines.

5   Once bacteria have been engineered in accordance with the invention, they may be grown in culture and the culture recovered to provide bacteria for a vaccine composition. Conditions for the growth of bacteria are well known in the art, and industrial processes for recovery of bacteria are also widely available.

10   ## Vaccine preparations.

The bacteria of the invention, or the membrane vesicles, may be formulated into vaccine preparations. Typically vaccines are prepared by mixing the bacteria with a pharmaceutically acceptable carrier or diluent. Such carriers or diluents include water, saline, dextrose, trehalose, 15   glycerol, ethanol or the like, or mixtures thereof. The vaccines may comprise one bacterial strain or a mixture of strains (i.e. either a mixture of different types of the same species and/or different species).

The concentration of the attenuated strain in the vaccine will be formulated to allow convenient 20   unit dosage forms to be prepared. Concentrations of from about 10^4 to 10^9 bacteria per ml will generally be suitable, e.g. from about 10^5 to 10^8 such as about 10^6 per ml. Where membrane vesicles are formulated, suitable concentrations will be in the range of from about 1 to 100, e.g. 5 to 50 mg of protein per ml.

25   The vaccine composition may be formulated as an aerosol, particularly for *N. meningitidis*, at concentrations the same as those described above.

Administration of vaccines.

30   The vaccines of the invention may be administered to recipients in order to protect them against diseases caused by the corresponding wild type organism. Although human recipients are primarily envisaged - especially for *N. meningitidis* which causes disease in humans - vaccines

of the invention will have veterinary uses, for example in vaccinating domestic pets such as cats and dogs, and in vaccinating livestock such as mammals including cattle, sheep and swine or other vertebrates such as poultry. These animals may be vaccinated in particular with attenuated enteric bacteria of the invention.

5

The vaccine may be administered by any suitable route. In general, subcutaneous or intramuscular injection is most convenient. This will lead predominately to a serum immune response which should prevent dissemination of the organism, throughout the body and the resulting septicaemia

10

Because it is unknown whether this route of administration will provide any protection against colonisation of the nasopharynx and carriage, vaccinations against bacteria which also invade through this route may also be by use of an intranasal spray. This will lead to colonisation of the nasopharynx mimicking closely the natural route of infection and carriage. The expected immune responses will be (a) mucosal - which would generate mucosal antibodies and should prevent colonisation by pathogenic bacteria and (b) serum - which should prevent dissemination of the bacteria throughout the body and the resulting septicaemia. This route of administration is particularly preferred for vaccination with attenuated strains of *N. meningitidis*.

20 Indeed, for *N. meningitidis* it is intended that this vaccine strain primarily will be delivered as a nasal aerosol, allowing the strain to colonise the nasopharynx (the initial site of colonisation for the wild-type organism). Due to the *asd* and the *galE* mutations the strain will exhibit a limited invasion of the tissues and possibly entry into the blood stream before viability is lost through serum killing and autolysis. This will mimic closely the progression of the natural route of infection, producing mucosal and serum immunity. This may be boosted if desired by injection.

For infants where there may be some reluctance to vaccinate with a live attenuated vaccine, non-viable membrane vesicles may be used. As described above, such vesicles are naturally produced by *N. meningitidis* and can be harvested as a vaccine to be administered subcutaneously, intramuscularly or by nasal aerosol.

Doses of vaccine will ultimately be at the discretion of the physician, taking into consideration

the status (e.g. age, weight, disease status) of the recipient and the nature of the vaccine strain used. However some general guidance may be found by reference to the published literature.

For example, intranasal doses of *N. meningitidis* may be calculated by reference to animal
5   models such as those disclosed in Microbial Pathogenesis (1992) 12:415-420 or Canadian Journal of Microbiology (1984) 30:1022-1029. Suitable intranasal doses are around 10^5 to 10^8, e.g. about 10^6 or 10^7 organisms in a single dose.

Live attenuated organisms may be administered subcutaneously or intramuscularly at up to 10^8
10 organisms in one or more doses, e.g. from around 10^5 to 10^8, e.g. about 10^6 or 10^7 organisms in a single dose.

Membrane vesicles may also be administered subcutaneously or intramuscularly in an amount of about 25-100 mg of protein (depending on LPS content of vesicles) in 2-3 doses. (See for
15 example Infection and Immunity (1996) 64:2745-2751, Infection and Immunity (1995) 63:4642-4652 or Infection and Immunity (1995) 63:3531-3536).

The recipient may receive one or more doses, in the latter case the doses may be repeated at an interval of from one week to one or more months, e.g. 2, 3, 6, 12, 18 or 24 months.
20

The above doses are provided primarily with reference to adult humans. They may be scaled up or down in proportion to body weight for non-adults or for other species.

The following examples illustrate the invention.
25

EXAMPLES

Materials and Methods.

30 The following PCR primers were synthesised for use in the genetic construction of vaccine strains. The primers were designed to contain a restriction site for ease of manipulation of the amplified products.

PRIMERS

	Name	Gene	Sequence	Direction	Enzyme
5					

	aro1	*aroA*	`                 T  G`
			`GGAATTCGATTCCGATGATATCCGTCATATG`
			`       CAG    C  CG G   C   C`
				forward	*Eco*RI

10					

	aro2	*aroA*	`           C`
			`GGAATTCATCAGGAATATGATTCATATCCAT`
			`        G  G  G  G  G      G`
				reverse	*Eco*RI

15	asdA	*asd*	`ACAATGAAAGTAGAATTCGTCGGCTGG`	forward	*Eco*RI

	asdB	*asd*	`   C`
			`GAATGCGGAGATGAATTCGCCGCCCAT`
			`  G  C T`
				reverse	*Eco*RI

20	furD	*fur*	`CCCTGCTCACGTCGACCAG`	reverse	*Sal*I
	furE	*fur*	`ACGCGGTCGACGCTGCACG`	forward	*Sal*I
25	furF	*fur*	`AATACGCAATTGGATCCTGCTTGC`	reverse	*Bam*HI
	furG	*fur*	`GATATTGAATCATATGGAAAAATTC`	forward	*Nde*I
	galEA	*galE1*	`GTGATTTTGGATAAGCTTTGCAATTCC`	forward	*Hind*III
30	galEB	*galE1*	`CCAGCGCCATGAAGCTTCCATCAT`	reverse	*Hind*III
	lac1	*lacI*	`GACAGGATCCAATGGTGCAAAACC`	forward	*Bam*HI
35	lac3	*lacZ*	`AATCATGGTCATATGTGTTTCCTG`	reverse	*Nde*I

| recA1 | recA | CGGAATTCGGTCTGAAGCGGATG | forward | EcoRI |
| recA2 | recA | CGCAGCAGGAATTCCCGTTTATCG | reverse | EcoRI |

5 These primers were used for amplifying target DNA to obtain copies of wild-type genes from target starting strains of bacteria. To obtain target DNA, 80 ml of overnight culture were grown in broth and harvested in 2 x 50 ml polypropylene tubes. The bacteria were recovered and DNA obtained using a standard phenol-chloroform extraction protocol.

10 Target genes were amplified by PCR under appropriate conditions carried out for 35 cycles, with the final cycle having an extension at 72°C for 9.9 minutes so that all extensions are completed. The purity, size and amount or PCR product obtained from any amplification is determined simply by agarose gel electrophoresis. 10 µl of the PCR reaction are analysed by agarose gel electrophoresis on a 1% agarose gel and different size DNA fragments separated
15 and identified under UV light by staining with ethidium bromide. The size of the amplified DNA fragment was determined by comparison with known size markers. The amplified product was cloned into one of a range of vectors including pUC19, pCRII, PCRscript and pSP72.

20 The above techniques may all be found in standard reference books such as Sambrook et al.., Cold Spring Harbor, 1989.

Mutants were constructed in vitro by identifying restriction enzyme sites from the information obtained by the sequencing of the cloned PCR product. Once restriction site(s) in the desired
25 area(s) are identified, the cloned fragment is cut with the appropriate enzyme(s), removing a fragment from the centre of the cloned insert. The plasmid is then re-ligated and deletion derivatives identified by the reduction in size on agarose gels and by sequencing. This procedure is used to develop defined mutations in the genes aroA, galE, asd and recA.

30 Vectors used for transformation of N. meningitidis include uptake sequences to assist with uptake of the vector by the bacterium. These sequences comprise a 10-mer inverted repeat Proc. Natl. Acad. Sci. (1988) 85:6982-6986). The nucleotide sequence of the uptake sequence

used is shown below, with the inverted repeats underlined in bold.

Uptake oligo   GGGCCCGGGCTGCA**GCCGTCTGAA**ATGCAT**TTCAGACGGC**TGCAGCCCGGGCCC

5   Transformation of *N. meningitidis.*

N. meningitidis grown overnight (37°C, 5% CO_2) on GC media (Difco) supplemented with 1% Vitox (Oxoid), are gently resuspended in protease peptone broth (15 g/l protease peptone No.3 (Difco), 4 g/l K_2HPO_4, 1 g/l KH_2PO_4, 5 g/l NaCl, 1% vitox) containing 10 mM $MgCl_2$ and

10 diluted to an OD_{600} of 0.2. 0.5 μg of plasmid is added to 0.5 ml of resuspended bacteria and incubated (37°C, 5% CO_2) for 4 hours. 100 μl aliquots are plated out on selective plates (GC agar containing antibiotics, and any other nutritional supplements required; see table below). Plasmids used for transformation are suicide vectors with various selectable markers (see table) and a synthetic uptake sequence specifically required for natural transformation of *Neisseria* sp.

15

Table: Media supplements for selection of mutants

Supplement	Working conc.	Use
Chloramphenicol	12 μg/ml	Selection of *cam* containing mutants
Erythromycin	5 μg/ml	Selection of *ery* containing mutants
Kanamycin	MIC*	Selection of *kan* containing mutants
Sucrose	15%	Selection of *SacRB* deficient mutants
Fe nitrate/sulphate	25 μM	Optional for *fur/lac* mutants
IPTG	0.1mM	Optional for *fur/lac* mutants
Diaminopimelic acid	100 μg/ml	Supplement for *asd* mutants

25

*Minimum inhibitory concentration

The final vaccine strain should preferably not contain any genes encoding antibiotic resistance. These genes are used as a marker of recombination events and may be removed by subsequent

30 recombination.

Following introduction of the mutants into host bacteria the introduction may be confirmed by

Southern blotting techniques under standard conditions, using the PCR amplified gene as a probe.

Example 1: _asd_ mutant.

5

A fragment comprising approximately 90% (1.3 kb) of the _asd_ gene was amplified using the Polymerase Chain Reaction (PCR). Primers asd1 and asd2 were designed by back-translation of the protein sequence based on the codon usage of _N. meningitidis_. _Eco_RI sites were included at the 5' ends of the primers to facilitate cloning into the plasmid pUC19. The resulting plasmid

10 pASD-1 was sequenced to confirm the identity of the cloned fragment and to generate a restriction map. Restriction sites were identified to create a deletion in the middle of the gene which also throws the latter part of the gene out of frame. The insert removed was a _Bcg_I fragment from positions 471-586 of the gene (where 1 is the A of the ATG start codon). This ensures complete inactivation of the gene product. A selectable marker cassette (comprising

15 either CAT and _Sac_RB genes which confer chloramphenicol resistance and sucrose sensitivity, or Erm and Rps1 genes which confer erythromycin resistance and streptomycin sensitivity (Johnston, 1996, pages 37-38 in Abstracts of the 10th International Pathogenic Neisseria Conference: Sept. 8-13 1996, Baltimore, MD, USA; eds. Zollinger _et al._)) was inserted at an _Nsp_I site of the undeleted _asd_ gene (this occurs within the _Bcg_I deletion site). This construct

20 was then used to transform _N. meningitidis_, with positive selection of mutants (resistance to chloramphenicol or erythromycin). Resistant colonies were then purified and transformed for a second time using the deletion construct devoid of any markers, with positive selection using either resistance to sucrose or streptomycin.

25 Mutants which have a mutation in the _asd_ gene which encodes the essential cell wall enzyme β-semialdehyde dehydrogenase can be identified by their requirement for diaminopimelic acid (DAP). Putative _asd_ mutants were replica plated onto DAP-deficient and DAP-replete media. Those strains which grew on DAP-replete medium (100 μg/ml) but not on DAP-deficient medium were _asd_ mutants.

30

Figure 1 illustrates schematically the construction of an _asd_ mutant in accordance with this example. Analogous schemes are described in the examples below for the production of other

mutants.

Example 2: *aro*A mutant.

5   Using procedures analogous to those of Example 1, an *aro*A mutant strain of *N. meningitidis* was constructed, via the use of the aro1 and aro2 primers listed above.

The *aro*A gene, which encodes the enzyme enolpyruvylshikimate phosphate synthase, plays an essential role in many pathways in numerous bacteria. Of these, the most important are the

10   roles in enterochelin-mediated iron uptake and aromatic amino acid biosynthesis. Mutants in *aro*A are unable to synthesise two essential compounds, *p*-aminobenzoic acid (PAB) and dihydroxybenzoate (DHB) and therefore can be identified by replica plating onto PAB and DHB deficient and replete medium. Strains that do not grow on the deficient medium but do grow on the replete medium are identified as the required mutants.

15

Example 3: *gal*E1 mutant.

N. meningitidis contains two chromosomal *galE* gene sequences, *galE1* and *galE2* at separate loci. *galE1* is complete, expressing a functional gene product, while *galE2* is truncated and

20   lacks homology over the first third of the protein. Primers galEA and galEB were designed to areas which are not conserved between *galE1* and *galE2* to amplify a 1.5 kb fragment of *galE1*. A *Hind*III site was engineered into each primer to facilitate cloning into pUC19 After subcloning into the suicide vector pGITB (a pUC based vector with neisserial uptake sequence and lacking a *Bpm*I site in the inactivated ampicillin gene, a selectable marker cassette (see

25   Example 1 for alternatives) was inserted at a *Bpm*I site (very close to the *Bcg*I deletion site). This construct was then used to transform *N. meningitidis*, and mutants identified by a chloramphenicol or erythromycin resistant phenotype. Resistant colonies were then purified and transformed for a second time using the *GalE1 Bcg*I deletion construct devoid of any markers, with positive selection using either resistance to sucrose or streptomycin.

30

To establish a change in LPS type, LPS is prepared from mutants and parental wild-type *N. meningitidis* by a proteinase K digestion of OMPs method and analysed on 15% tricine SDS-

PAGE gels. Bands are visualised by silver staining. LPS from *galE* mutants demonstrate greater electrophoretic mobility than their wild-type parent strains.

Example 4: Δ*asd* Δ*galE* and Δ*aroA* Δ*galE* mutants

5

The procedure of Example 3 is repeated on the mutants produced by the processes described in Examples 1 and 2. This provides mutants which are Δ*asd* Δ*galE* and Δ*aroA* Δ*galE* mutants respectively.

10   Example 5: *fur* constructs.

For alterations in the *fur* gene it was essential not to have a complete knockout mutant since this may be lethal. Therefore the *fur* gene was placed under the control of another promoter which could be switched on or off independently of the factors (iron) which normally controls *fur* 15 expression.

Primers for the amplification of the entire *fur* gene were designed from the published sequence with an *Nde*I restriction site in the forward primer (primer furG) at the transcription start of the gene and a *Sal*I restriction site in the reverse primer (primer furD) at the distal end of the *fur* 20 gene. Primers furD and furG were used for the amplification of a 600 bp fragment containing the entire *fur* coding sequence using PCR. The DNA fragment was polished with *Pfu* polymerase and cloned into the vector pCR-script (Stratagene), to give the plasmid pFUR-DG. Primers for the amplification of a 300 bp region upstream of the *fur* gene were designed with a *Bam*HI restriction site in the reverse primer (primer furF), proximal to the transcription start and 25 a *Sal*I restriction site in the forward primer (primer furE), distal to the transcription start. The 300 bp DNA fragment was cloned into the vector pCR-script as above to give the plasmid pFUR-EF.

Finally, two primers were designed and constructed to the *lacZ* promoter and repressor region 30 (*lacI*) of the *E. coli lac* operon. The forward primer, lac1, contained a *Bam*HI site and the reverse primer, lac3, included the *Iq* mutation and an *Nde*I site which would regenerate the ATG transcription start when ligated to the *Nde*I site of the gene pFUR-DG plasmid insert. The

fragment generated using these primers was cloned into the vector pCRII (Invitrogen), to give pLAC-13.

The Lac-13 fragment was removed from its vector by cutting with *Nde*I and *Bam*HI, the fur-1
5   fragment with *Bam*HI and *Sal*I, the Fur-DG fragment with *Nde*I and *Sal*I. These were sequentially ligated together to give the complete construct, pFLF in the vector pCRscript (Stratagene). This was then sub-cloned into the suicide vector and inserted into the chromosomal location of the wild-type *fur* gene by allelic exchange.

10   To demonstrate that fur expression is under *lac* control in the *fur/lac* mutant, IPTG was added to iron-rich growth medium and the bacteria analysed for an iron-restricted protein profile.

Analysis of the *fur* mutants by observing changes in iron regulated OMPs (Outer Membrane Proteins) can be performed using a very convenient phenotypic assay. OMPs prepared from *fur*
15   mutants and wild types show that *fur* mutants are deregulated for certain iron regulated proteins. To ascertain this cells are grown overnight in culture to A_{450} = 0.5 and harvested by centrifugation.   Cells are suspended, sonicated and the membranes precipitated from the supernatant and recovered. To confirm the identity of the over-expressed OMPs, proteins are transferred by electroblotting onto nitrocellulose membranes and probed with specific
20   antibodies to known fur-regulated proteins e.g. tbp1, tbp2, fbp (37 kDa), and frp (70 kDa).

Example 6: Δ*asd*, Δ*galE*, *lac:fur* fusion and Δ*aroA*, Δ*galE*, *lac:fur* fusion mutants.

The procedure of Example 5 was repeated using the Δ*asd*, Δ*galE* and Δ*aroA*, Δ*galE* mutants
25   described in Example 4 to produce Δ*asd*, Δ*galE*, *lac:fur* fusion and Δ*aroA*, Δ*galE*, *lac:fur* fusion mutants respectively.

Example 7: *rec*A mutant.

30   DNA sequences of *rec*A genes from various bacterial species were compared and primers recA1 and recA2 were designed within conserved regions of the gene using the codon frequency of *N. meningitidis*. The primers were designed with *Eco*RI restriction enzyme cleavage sites to

facilitate cloning. The primers were used to amplify *rec*A from *N. meningitidis* and the product was cloned into pUC19. The resulting plasmid, pRECA-1, was partially sequenced to verify that the insert was from the *rec*A gene and restriction mapped to identify useful restriction enzyme sites for the generation of the deletion derivative. This deletion derivative, pRECA-D,

5   was recombined into the wild type *N. meningitidis* chromosome and the resulting strain was tested to determine its sensitivity to UV light compared to the parental strain.

UV sensitivity compared to the isogenic wild type strain can be determined by inoculating blood agar plates with parallel streaks of the wild type and the putative *rec*A mutant. The

10 inoculated plates are exposed to known UV energy for increasing times. The plates are incubated at 37°C and 5% CO_2 in air for 24 hours. UV sensitivity is shown by reduced growth of the putative mutant on plates compared to the adjacent wild-type strain.

The strains obtained by this procedure are effectively isolated genetically and no further

15 construction can be undertaken since they are now deficient in homologous recombination.

Example 8: Δ*asd*, Δ*gal*E, *lac:fur* fusion, *rec*A and Δ*aro*A, Δ*gal*E, *lac:fur* fusion, *rec*A mutants.

The procedure of Example 7 was repeated, but this time using strains of *N. meningitidis*

20 obtained by the procedure of Example 6. This provides Δ*asd*, Δ*gal*E, *lac:fur* fusion Δ*rec*A and Δ*aro*A, Δ*gal*E, *lac:fur* fusion Δ*rec*A mutants respectively.

CLAIMS

1.   An attenuated bacterium in which the native *fur* gene, or homologue thereof, is modified such that the expression of the *fur* gene product, or homologue thereof, is regulated independently of the iron concentration in the environment of the bacterium.

2.   A bacterium according to claim 1 which is a gram-negative bacterium.

3.   A bacterium according to claim 2 which is selected from the group consisting of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Helicobacter pylori*, *Salmonella typhi*, *Salmonella typhimurium*, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), verotoxigenic *E. coli* (VTEC), *Vibrio cholerae*, *Shigella spp.*, *Haemophilus influenzae*, *Bordetella pertussis* and *Pseudomonas aeruginosa*.

4.   A bacterium according to claim 3 which is selected from *Neisseria meningitidis* and *Neisseria gonorrhoeae*.

5.   A bacterium according to any one of the preceding claims which has been attenuated by mutation of a gene essential for the production of a metabolite or catabolite not produced by a human or animal.

6.   A bacterium according to any one of the preceding claims which has been attenuated by a mutation of an *asd* gene and/or a gene of the *aro*, *pur*, or *pyr* pathways.

7.   A bacterium according to claim 6 wherein said gene is *asd* or *aro*A.

8.   A bacterium according to any one of the preceding claims which further comprises a *rec*A mutation.

9.   A bacterium according to any one of the preceding claims which further comprises a mutation in which expression of a toxin gene has been modified or eliminated.

10. A bacterium according to any one of the preceding claims which further comprises a mutation at a site homologous to the *E. coli minA/minB* locus.

11. A bacterium according to any one of the preceding claims which further comprises a mutation in a gene involved in the uptake of DNA.

12. A bacterium according to claim 11 which is *N. meningitidis* or *N. gonorrhoeae* and wherein the gene involved in the uptake of DNA is *com*A.

13. A bacterium according to any one of the preceding claims which is *N. meningitidis* or *N. gonorrhoeae* and which further comprises a mutation in the *gal*E gene.

14. A bacterium according to claim 13 which further comprises a mutation in the *opc* gene to modify or eliminate expression of opc protein.

15. An *N. meningitidis* strain which has the genotype: Δ*asd*, Δ*gal*E, *lac:fur* fusion, Δ*rec*A.

16. An *N. meningitidis* strain which has the genotype: Δ*aro*A, Δ*gal*E, *lac:fur* fusion, Δ*rec*A.

17. An *N. meningitidis* strain according to claim 15 or 16 which further comprises at least one of a *minA/minB* mutation, an RTX negative phenotype, and an *opc* gene the expression of which has been modified or eliminated.

18. A preparation of membrane vesicles obtained from a bacterium as defined in any one of claims 1 to 17.

19. A vaccine preparation which comprises a bacterium as defined in any one of claims 1 to 17 or a preparation as defined in to claim 18 together with a pharmaceutically acceptable diluent or carrier.

20. A vaccine according to claim 20 for use in a method of treatment of the human or

animal body.

21. A method of protecting an individual against a bacterial infection which comprises administering to the individual an effective amount of a vaccine composition as defined in claim 19.

22. A process for preparing a vaccine composition comprising an attenuated bacterium as defined in any one of claims 1 to 17 which process comprises:

(a) inoculating a culture vessel containing a nutrient medium suitable for growth of said bacterium;

(b) culturing said bacterium;

(c) recovering bacteria from the culture; and

(d) mixing said bacteria with a pharmaceutically acceptable diluent or carrier.

23. A method for producing a bacterium according to claim 1 which method comprises modifying the native *fur* gene, or homologue thereof, of an attenuated bacterium such that expression of said *fur* gene or homologue is regulated independently of the iron concentration in the environment of the bacterium.

24. A method according to claim 23 wherein said bacterium has been attenuated by at least one mutation as defined in any one of claims 5 to 7.

ABSTRACT

LIVE ATTENUATED VACCINES

The present invention provides an attenuated bacterium in which the native *fur* gene, or homologue thereof, is modified such that the expression of the *fur* gene product, or homologue thereof, is regulated independently of the iron concentration in the environment of the bacterium.
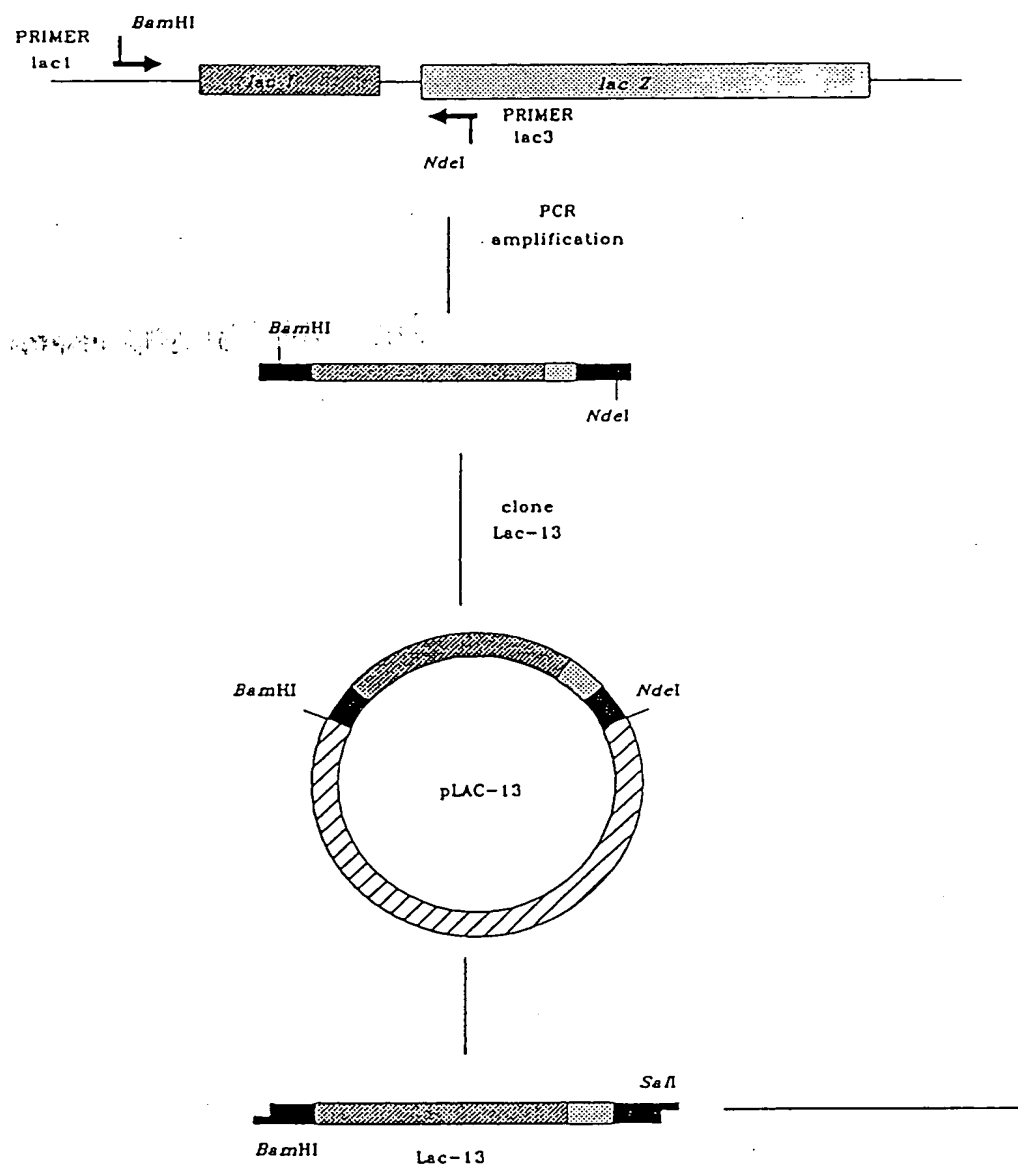
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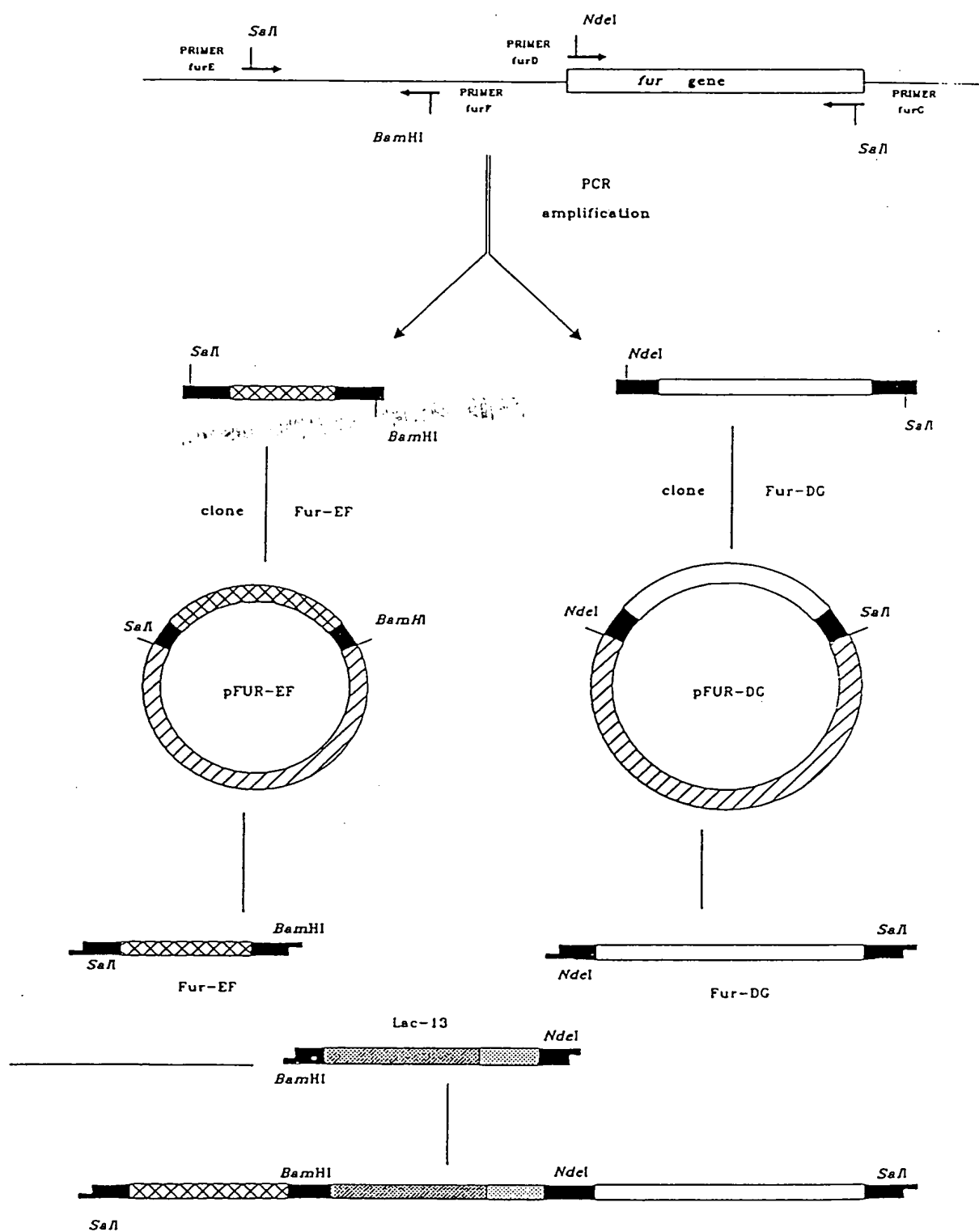
Figure 1. Construction of *asd* mutant
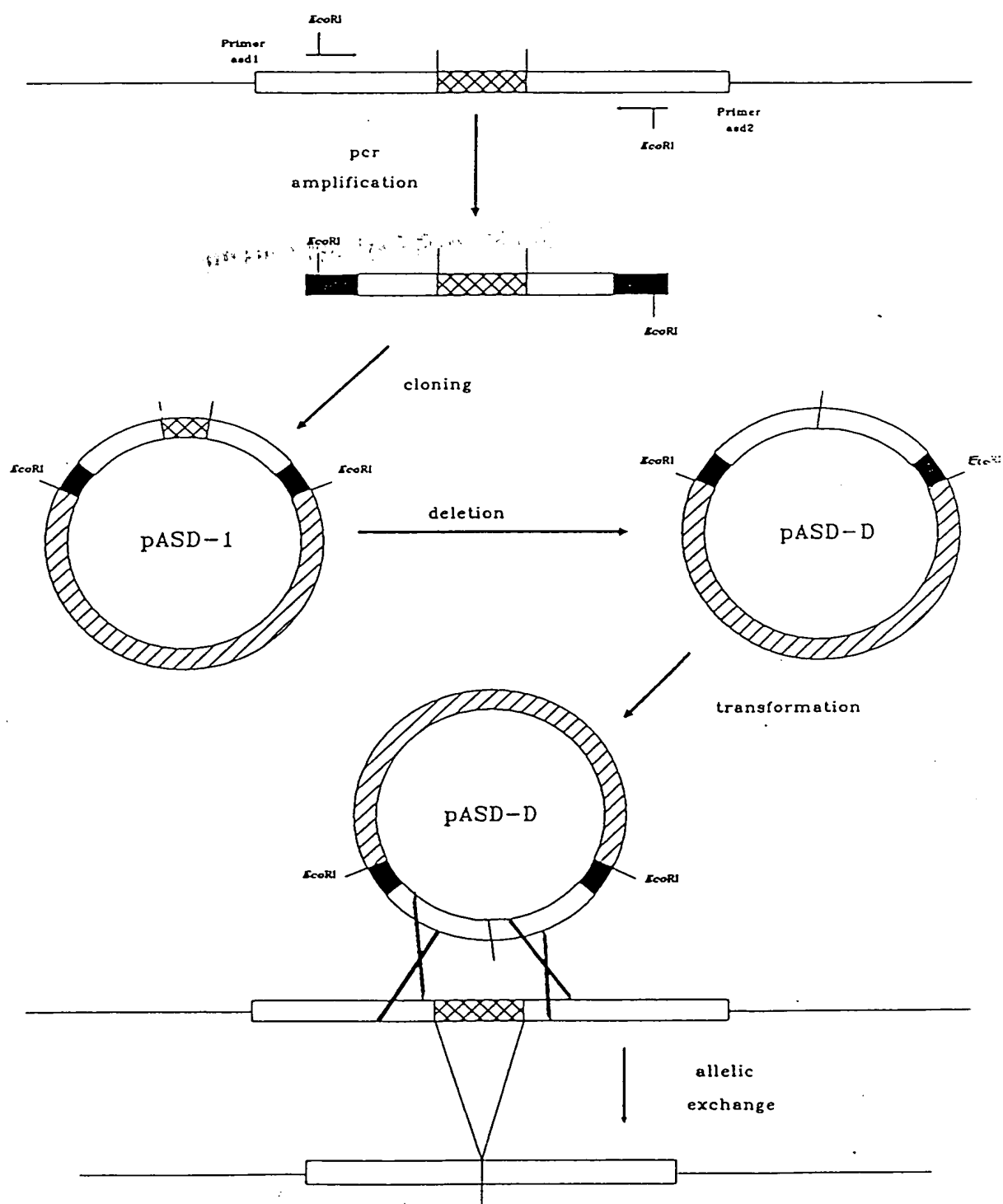
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Figure   2a   Construction of *fur/lac* fusion

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Figure 2b Construction of *fur/lac* fusion (continued)

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